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Treatment of Soy Milk Oligosaccharides by a Homogeneric Enzyme Extract Containing A-Galactosidase.

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TREATMENT OF SOY MILK OLIGOSACCHARIDES BY A HOMOGENERIC ENZYME EXTRACT CONTAINING α-GALACTOSIDASE

A DISSERTATION

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in

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by
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DEDICATION

To

HELEN SHANNON CROCCO, ALBERT RICHARD CROCCO

and JOHN F. SHERIDAN, S.J.

who taught me to dream the impossible dream

AND TO ALL THOSE WHO HELPED, WHENEVER MY IMPOSSIBLE DREAM TURNED INTO MY IMPROBABLE NIGHTMARE,

especially

Jeanne, Steven, Dirk and Jenné Graeser, a group of Grand Southerners who cared about their Yankee.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Intestinal flatulence is reputed to be a usual sequel to the ingestion of legumes of the bean family. Treatment of soy milk oligosaccharides by purified preparations of the enzyme α-galactosidase from microbial or fungal sources is an experimentally sophisticated but relatively expensive means of "pre-digesting" the oligosaccharides incriminated in this flatulence.

The potential use of a crude enzyme extract from the germinating seeds of the soybean Glycine max, var. Pickett, was studied. Paper chromatograms developed for 18 hrs showed degradation of the oligosaccharides found in commercial soybean milk with a concomitant increase in the amount of mono- and disaccharides present. All of the simpler sugars present were identified with the exception of one. A tentative identification of that sugar was discussed.

The properties of the crude enzyme extract obtained from the germinating soybeans which would make the preparation desirable from the industrial standpoint were also examined. In this preparation, the enzyme α-galactosidase was stable to the repeated effects of cyclic freezing and thawing. Furthermore, the activity of the enzyme

was not significantly affected by the process of lyophilization. Moreover, the latter process could be used to concentrate the enzyme preparation.

Studies on the relationship of pH to activity showed that the optimum pH was achieved in a phosphate buffer system at pH 4.6. There was some indication that phosphate buffer was a better suspending menstruum than an analogous citrate system. The optimum pH range of the α-galactosidase preparation compared favorably with those reported by other investigators.

An examination of data reported in relation to the needs of the food industry was presented. Some suggestions were made as to the future use of the proposed scheme involving the enzyme preparation studied.

INTRODUCTION

Leguminous foods of the bean family and their processed food products and food substitutes have, for years, been regarded as flatus-inducing foods. Gas produced in the large intestine as a result of their ingestion can cause acute intestinal upset and discomfort, especially in the young and the aged. Populations of the emerging nations find some bean products unacceptable not because of nutritional or taste standards, but rather due to the flatus-related discomfort resulting from their ingestion.

With the human population still growing logarithmically in many areas and the concomitant need for an increase in foods for this population, attention has been paid to the direct and indirect use of soybeans and their products in human nutrition. Soybeans, at present, are directly used as infant hypo-allergenic milks or as protein sources for addition to other forms of food. Soybeans are an excellent source of protein supplement in diets for livestock intended for slaughter.

If the assumption is correct that the soybean or its processed products contains inherently a substance which causes the induction of flatus, then the increased need for and use of soybeans, soy flours and other soy products will

cause an unfortunate increase in the associated phenomenon of flatus. This can render an otherwise nutritionally acceptable product as commercially unsuccessful.

In view of the world-wide protein crisis, there is a need for the obviation of the undesirable side-effects of the ingestion of flatus-inducers such as soy products. This should enhance the manufacture of more desirable commercial protein products as well as a more desirable primary soy product. This is especially true of the commercial soybean milks prepared for infant feeding, since additional colic in an already distressed and allergic infant results in problems for both mother and child. The elimination of the cause of flatulence in commercial soybean milks and other products will result in a more acceptable substitute for the more traditional meat proteins in human nutrition.

One of the characteristics common to all legumes is the presence of oligosaccharides of the raffinose family, i.e., raffinose, stachyose, verbascose (Figure 1), as cotyledon storage forms of the monosaccharides needed for germination and early seed growth. Although there is, at present, no direct evidence that raffinose-type oligosaccharides are the sole cause of flatulence in human beings when the bean or its product is ingested, there is indirect evidence to support this hypothesis [12, 17].

Consequently, if a system could be developed which would "pre-digest" some of the incriminated oligosaccharides,

RAFFINOSE

α-D-Gal-(1——6)-α-D-Glu-(1——2)-β-D-Fru

\+ α-D-Gal

STACHYOSE

α-D-Gal-(1——6)-α-D-Gal-(1——6)-α-D-Glu-(1——2)-β-D-Fru

\+ α-D-Gal

VERBASCOSE

α-D-Gal-(1——6)-α-D-Gal-(1——6)-α-D-Gal-(1——6)-α-D-Glu-(1——2)-β-D-Fru

Figure 1. Structural relationships of some common sugars of the raffinose family showing the number and extent of the α-galactosidic bonding

and thus render the substance less likely to induce flatulence, the economic and nutritional desirability of the product would be increased as a direct source of human food.

Ideally, a purified enzyme with a suitable structure might be found which could be bound to an inert substrate such as porous glass to form a solid site for chemical reaction [3]. In this fashion, the enzyme could be made available to its substrate, in this case the α-bonded galactosides of soy milk, in a continuous flow process with little or no loss of enzyme. If such a scheme were possible, then the method and time involved in the purification and concentration of the enzyme to be used would not matter, since the enzyme would not need to be isolated, purified and concentrated continuously. Thus any one or a combination of methods cited in the literature for this process of enzyme purification could be used.

The scope of this work, however, was to isolate the enzyme α-galactosidase (E.C.3.2.1.22) in a commercially feasible manner, not necessarily in the pure, crystalline form needed for an enzymological study. For this reason, a study of the enzyme α-galactosidase was undertaken using simple, quick and relatively inexpensive means to produce large amounts of an enzyme which would cleave the α-bonds of the oligosaccharides found in soy milk, prior to the actual commercial milk processing. Such a scheme would allow the processor to reduce the oligosaccharide content of the soy product with a homogeneric enzyme preparation

without the economic need to re-cycle the enzyme preparation for subsequent soy milk processing. Additionally, extraneous, heterogeneous protein would not be introduced into the soy system, which could be of importance, particularly in the hypo-allergenic milks.

REVIEW OF THE LITERATURE

Prior to 1961, there was little mention of the enzyme α-galactosidase although oligosaccharides of the raffinose family, with α-D-galactosidic bonds were well known to be widely distributed in nature. The 1961 review of Wallenfels and Malhotra [39] which devotes 59 pages to the galactosidases indeed gives only 9 of those pages to the α-galactosidases. This review indicates, at best, uncertain knowledge of even the most basic properties of the α-galactosidases in comparison with the well-known characterization of the β-galactosidases from various sources.

Since 1961, much work has been done on the occurrence and properties of the α-galactosidases from various sources, due in part to the ability of workers to obtain the enzyme in pure form. Table 1 lists the sources cited for purified, partially purified or observed α-galactosidase, together with the date of the report in the literature.

Purification of the Enzyme

In 1963, Li et al. [24] reported the isolation and purification of α-galactosidase from the culture media of Diplococcus pneumoniae. Some of the properties of the enzume were also discussed. This purification scheme,

TABLE 1

Source, General Biological Type, Date and Literature Citation for Some Recently Reported α-Galactosidases

Source	Biological type	Date	Cited as
Diplococcus pneumoniae	bacteria	1961	22,24
Streptococcus bovis	bacteria	1963	6
Epidinium ecaudatum	protozoa	1963	7
Calvathia cyathiformis	fungus	1964	23
Streptomyces olivaceus	bacteria	1966	37
Sacromyces sp. (hybrids)	yeasts	1966	21
Coffea sp.	coffee seeds[1]	1968	31
rat (uterus)	mammal	1968	11
Phaseolus vulgaris	bean seeds	1968	1
sugarcane (invertase)	plant	1969	2
Aspergillus niger	mold	1969	5
jack bean	bean seeds	1969	10
sweet almond	plant	1969	14
Actinomycetales sp.	bacteria	1969	25
Vicia sativa	bean seeds	1969	30
Aspergillus saitoi	mold	1970	33
Mortierella vinacea	fungus	1970	34
Acer pseudoplatanus	plant	1970	19
spinach leaves	plant	1970	16
Calletotrichum sp.	fungus	1970	18
Vicia faba	bean seeds	1971	8
Pisium sativium	bean seeds	1971	8
Escherichia coli, K-12	bacteria	1971	9
barley	plant	1972	27

[1]General biological type for seeds is plant. The nature of this paper made the specific listing of each seed α-galactosidase preferable.

however, requires three successive ammonium sulfate precipitation steps, several days, and the absorption of the enzyme neuriminidase by human red blood cells. As such, Diplococcus pneumoniae is unsuitable as an industrial source of the enzyme, regardless of yield.

Li and Shetlar [23] reported the isolation, purification and partial characterization of α-galactosidase from the puffball Calvatia cyathiformis. The purification involved two successive acetone precipitations, dialysis and fractionation by carboxymethylcellulose (CMC) column chromatography. Further purification of active fractions was effected by ultrafiltration. This method, too, involves several successive steps and several days.

A partially purified α-galactosidase was prepared from the culture broth of Streptomyces olivaceus as reported by Suzuki et al. [37] but again, the steps involved were time consuming, tedious and not suited to industrial needs.

Other authors [1,5,10,11,13,16,19,26,30,34] have described methods of purification, all having in common at least one fractionation with high concentrations of $(NH_4)_2SO_4$ and the use of column chromatography to further purify the partially purified enzyme preparations. These literature reports are summarized in Table 2. The time and expense required for such schemes is industrially unacceptable, considering the volume of material which would need to be subjected to fractionation and purification.

TABLE 2

Literature Citation and Enumeration of Steps Involved in Various Purification Schemes for the Enzyme α-Galactosidase

Literature citation	Purification schemes
1	$(NH_4)_2SO_4$ pptn.; DEAE-Sephadex, CM-Sephadex chromatography
5	$(NH_4)_2SO_4$ pptn.; ultrafiltration; Sephadex G-150, DEAE-Sephadex chromatography
10	$(NH_4)_2SO_4$ pptn.; Sephadex G-100 chromatography; ethanol fractionation
11	$(NH_4)_2SO_4$ pptn.; Bio-Gel P-200, CMC chromatography
13	pH change; protamine sulfate pptn.; acetone fractionation; dialysis (X5)[1], Sephadex G-100 chromatography
26	$MnSO_4$ pptn.; acetone fractionation; pH change, $(NH_4)_2SO_4$ pptn.; CMC (X2)[1], Sephadex G-75 chromatography
16	pH change; $(NH_4)_2SO_4$ pptn.; Sephadex G-150 chromatography
19	cell disruption; $(NH_4)_2SO_4$ pptn.; centrifugation; dialysis
30	$MnSO_4$ pptn.; centrifugation; $(NH_4)_2SO_4$ pptn.; dialysis; hydroxylapetite, Sephadex G-75 chromatography
34	$(NH_4)_2SO_4$ pptn.; DEAE-Sephadex, Bio-Gel P-200 chromatography; crystallization

[1]Refers to the number of times the procedure was repeated.

Moreover, commercial soy milks are often made from defatted soy flakes which have been rehydrated, homogenized, canned and then retorted [32]. In such a method, any enzyme added for the purpose of decreasing the number of α-bonds in the oligosaccharide content of the soy milk would be destroyed by the retorting process. New enzymes would be required for each new input of defatted soy flakes or soybeans in the manufacturing process. Thus, industrially, a somewhat different method for obtaining the needed enzyme is desirable.

Activity of Purified Enzyme Systems

The studies reported by several authors about the pH optima for the α-galactosidases is summarized in Table 3. The optimum pH or optimum pH range is almost universally acid. The optimum pH for α-galactosidase activity generally is somewhere between pH 3.0 and 6.0. These findings are consistent with the earlier report of Wallenfels and Malhotra [39] that α-galactosidase appears to have a "flat" optimum pH curve in the acid range. Thus it would appear that there is a basic similarity between the α-galactosidases, regardless of the specific source.

It should be noted that the optimum pH was found to be above 7.0 only for the α-galactosidase from *E. coli* K_{12} [9]. This fact was commented on by the authors as significant, although no explanation was offered for the observation. Additionally it was found that the α-galactosidase

TABLE 3

Source, Substrate and Optimum pH or Optimum pH Range for Some Recently Reported α-Galactosidases

Source	Substrate	pH
Epidinium ecaudatum	melibiose	5.5
Pisium sativum	PNPG[1]	6.3
Escherichia coli, K-12	PNPG	8.1
Mortierella vinacea	raffinose	4.0
Calvathia cyathiformis	ONPG[2]	3.0-5.0
Diplococcus pneumoniae	ONPG	5.6-6.0
Streptomyces olivaceus	melibiose	5.4
rat (uterus)	ONPG	5.2
Vicia faba	raffinose	3.5-5.5
spinach leaves	PNPG	5.3
Acer pseudoplatanus	PNPG	3.7 and 6.3
Streptococcus bovis	melibiose	5.6-6.3
Mortierella vinacea	ONPG	4.0-6.0
Aspergillus niger	PNPG	3.8-4.2
Phaseolus vulgaris	PNPG	6.5-6.7

[1]Paranitrophenyl-1-6-α-D-galactopyranoside

[2]Orthonitrophenyl-1-6-α-D-galactopyranoside

from this strain of E. coli had a requirement for nicotine adenine dinucleotide (NAD), a co-enzyme usually involved in oxidation-reduction. This observation also remained unexplained.

Freeze-Thaw Stability

Various authors who have studied the α-galactosidases have worked with the optimum temperature for the activity of the enzyme [6,7,23], or with the temperature of inactivation [1,2,5,7,13,16,23,24,29,30,33,35,36,37,38]. Only a few have discussed the stability of their preparation to low temperature. No evidence has yet been presented for the stability of an α-galactosidase preparation to repeated cycles of freezing and thawing.

Some investigators [5,9,11,34] stated that their enzyme preparation was not stable to freezing and thawing. The purified enzyme preparation of Bahl and Agrawal [5] from Aspergillus niger was not only unstable to freezing and thawing but could be successfully freeze-dried only in the presence of 5 mg/ml ammonium sulfate. Burstein and Kepes [9] found that repeated freezing and thawing causes a precipitation of protein and the concomitant loss of enzyme activity. Moreover, they stated that the storage of the crude enzyme extract for five days at 4°C results in a 95% loss of activity. Alpha-galactosidase from rat uterus [11] lost activity very rapidly at room temperature and also upon freeze-thawing.

There is some disagreement with the reported results, however, since the α-galactosidase from D. pneumoniae [24] was reported as stable when kept in a frozen state. Implicit in this statement is the concept that this particular preparation is stable to at least one freeze-thaw cycle, although no mention is made of the attempt to study freeze-thaw stability per se.

The α-galactosidase from spinach leaves [16] was stored after purification at -20°C and retained full activity for one month when stored at this temperature. Moreover, the purification of α-galactosidase from the seeds of Vicia faba [8] required acetone fractionation at -20°C, implying the cryostability of this enzyme. It is perhaps significant that in these two latter indications of cryostability, the original source of the enzyme was from plant tissue. Although it is not mentioned as significant, it is generally accepted that plants must be able to withstand some abrupt changes in temperature, with the implication that their enzyme systems are thus stable to these abrupt changes of temperature.

The stability of any industrial preparation to more than one freeze-thaw cycle is important for the obvious reasons of work schedules and processing cycles.

Freeze-Dry Stability

Many investigators working with α-galactosidase for commercial applications failed to mention any relationship between their enzyme preparation and the technique of lyophilization. The latter is an industrially feasible process for the concentration of an enzyme preparation.

Alexander [2] implied the stability of the *in vivo* enzyme by using lyophilized, immature storage tissues of 16-week-old plants as the source of his preparation. The fact that the enzyme was recoverable from this source in an active state indicates that, at least within the tissues, the enzyme α-galactosidase is stable to the effects of the freeze-dry process.

Burstein and Kepes [9] indicated the potential use of the freeze-dry process to concentrate an enzyme preparation by stating that inactivation by dilution could be reversed by freeze-drying the diluted enzyme preparation and re-dissolving the dry pellet in a smaller volume. This is, in essence, a definition of the technique of concentration by lyophilization. These authors, who prepared what was referred to as an "incomplete report" because of the instability of their preparation, nevertheless ascertained that their preparation was stable to the freeze-drying process without any special protective measures.

However, Bahl and Agrawal [5] stated that their enzyme preparations were unstable to freeze-drying unless they were dried in the presence of ammonium sulfate, hence their

solutions were concentrated by ultrafiltration.

The technique of concentration by lyophilization is not only industrially feasible, but also has the advantage of allowing a processor to add an enzyme to a solution in a powder form. Re-hydration occurs in the very solution in which the enzyme is to act. The advantages of such a system in the industrial realm would more than outweigh the original cost of the equipment involved.

Action on Oligosaccharides

Although the solvent and paper systems used in chromatography may differ from author to author depending on which compounds are to be separated, the majority of the workers have found a number of common properties among the oligosaccharide-enzyme systems studied.

Whether the system utilized butanol:pyridine:0.1N HCl [22,23,24], butanol:pyridine:H_2O [1,9] or propyl alcohol:ethylacetate:H_2O [36] the systems involved clearly demonstrated several characteristics of an α-galactosidase catalyzed reaction involving oligosaccharides of the raffinose family.

The enzyme α-galactosidase was clearly shown to hydrolyze the terminal α-1-6-galactosidic bond of any sugar which possesses such a bond. It is significant that all the known oligosaccharides of this family, some of which are shown in Figure 1, have this characteristic. Additionally, α-galactosidase was reported [37] and demonstrated

[2,22,23,24,33] to have a transglycolase property as well as that of a galactosidase, per se. In systems involving several of these related sugars, intermediates of such a galactosidase-transglycolase reaction could be demonstrated. Chromatographs of substances involved in reactions stopped at different time intervals showed the accumulation of certain products involved in both galactosidase and transglycolase reactions. These were present in increasing concentrations with the concomitant decrease in the "parent sugar."

In general, when chromatographs were developed, a system was chosen to demonstrate the presence of reducing sugars. In some instances [2,23,24] the detecting reagent was chosen to demonstrate the presence of the non-reducing sugar raffinose. There was some disagreement as to whether or not the diphenylamine-aniline-phosphate detecting reagent demonstrated only the presence of the reducing sugars [9,22] or was able to detect both reducing and non-reducing sugars [33].

In the published chromatograms, stachyose was shown to move slowly [2,22,23,24,33]. However, in any mixture of sugars in which stachyose was a component, there was effected a separation of stachyose from the other components by the comparative immobility of stachyose in relation to the other sugars in the system.

MATERIALS AND METHODS

Several different strains and species of yeasts were used in preliminary studies to test for their production of the enzyme α-galactosidase. It was thought that among them could be a prolific producer of the enzyme which could be used in the treatment of soy milk and other soy products and substrates.

Maintenance Medium

The yeast cultures were maintained on M-12a basal agar medium composed of 2% dimalt, 0.5% peptone, 0.3% yeast extract, 1.5% agar (w/v) dissolved in distilled water. The medium was autoclaved at 121°C for 15 min and cooled as slants.

Growth Media

Prior to testing against specific α-bonded substrates, the cultures were transplanted from the basal maintenance medium into a broth composed of Yeast Nitrogen Base (YNB, Difco) with glucose added in a concentration of 0.1% (w/v) as the sole source of carbohydrate. The cultures were grown for 24 hours in a roller drum (model TC-5, New Brunswick Scientific Co., 60 rpm). When turbidity was obvious, the cultures were transplanted in amounts of 0.5

ml into a "second stage" medium of the same composition as the above. After 24 hours, the cultures were transplanted into a challenge medium, described below. Again, 0.5 ml was used as the inoculum.

Challenge Medium

Cells were transferred to YNB broth containing raffinose in the concentration of 1.0% (w/v). The cultures were placed in a roller drum and allowed to grow for 24 hours. Following growth and a time interval for sedimentation, the supernatants were tested for the presence of α-galactosidase using the specific substrate orthonitrophenyl-1-6-α-D-galactopyranoside (α-ONPG). The reaction was stopped after 15 min by the addition of sodium carbonate and the absorbance of the liberated orthonitrophenyl was determined using spectrophotometric means. This was considered as a routine assay procedure with the specifics of the procedure listed below. Cultures with negative reactions on first challenge were discarded as unproductive.

Routine Assay Procedure

Assays were routinely performed to periodically check for the presence of the enzyme α-galactosidase. A working solution of α-ONPG (see below) was maintained at 4°C so that any given solution could be readily tested. Routine assays were performed on all test solutions by taking 1 ml of sample and combining it with 1 ml of the working solution of α-ONPG. After incubation in a water

bath at 37°C for 15 min, the reaction was terminated by the addition of 2.0 ml of aqueous sodium carbonate solution (0.25 M). The final 4 ml volume was then examined spectrophotometrically with a Bausch and Lomb Spectronic 20, set at 400 mμ. With some routine assays, the production of o-nitrophenyl was recorded as + or -, in other instances percent transmittance was recorded. Since all volumes from each assay were the same, transmittances, when recorded, could be compared between solutions.

Additional Media

Several other media were used in the attempt to induce and/or recover α-galactosidase from cultures of yeast cells. Erlenmeyer flasks containing 100 ml of lactose broth (Difco) with additional lactose sufficient for final concentrations of 2.5 and 5% (w/v) were inoculated with 5.0 ml of a "second stage" culture in YNB with glucose (GluYNB). The cultures were incubated for several days on a shaker and samples were routinely tested for the presence of α-galactosidase with α-ONPG (as above).

Another medium consisted of aqueous whey broth and was produced by the treatment of a commercial whey product, MNC Whey (Milk Nutrients Concentrated, partially delactosed whey, Foremost Foods Co., San Francisco, California). The aqueous whey solution was made by suspending 10 g of whey and 20 g of lactose in one liter of distilled water. The temperature of the solution was raised to 80°C

and maintained at that temperature for 15 min using a magnetic stirrer/hot plate combination. The liquid was allowed to cool and then centrifuged in a Sorvall RCB-2 refrigerated centrifuge for 20 min at 6000 rpm. The supernatant was then used as a culture medium and was dispensed into flasks in 100 ml amounts. Corresponding solutions were made using phosphate buffer, pH 7.0, as the suspending menstruum. All solutions were sterilized at 121°C for 15 min.

Analogous preparations were made using raffinose at a concentration of 2.0 and 5.0% (w/v) in place of the added lactose. As with broths enriched with additional lactose, the raffinose preparations were done with both distilled water and phosphate buffer (pH 7.0) as the suspending liquid, and sterilization was effected by autoclaving.

Freeze-Concentration

The removal of water from supernatant culture fluids by ice-crystal formation was studied as a possible means for the concentration of solutions containing α-galactosidase. Solutions were partially frozen, stirred with a glass rod, then filtered through cheesecloth. The ice crystals which were separated by the cheesecloth were discarded. The solution resulting from this sieving was rechilled and subjected to a second, similar treatment. When the volume of the original solution was reduced by at least 50%, the concentrate was tested with ONPG in the routine manner.

Residual Carbohydrate Studies

The amount of carbohydrate present at various stages was determined by the phenol-sulfuric acid method of Dubois et al. [15]. To avoid the use of micro-pipettes, the phenol was added as a 5% (w/v) solution in distilled water. The amounts of the reactants involved were: 1 ml of test solution, 1 ml 5% phenol in water and 5 ml of concentrated sulfuric acid.

The test and phenol solutions were combined and thoroughly mixed in Pyrex test tubes. Sulfuric acid was added by a quick-delivery graduated buret and the system was allowed to cool as recommended by Montgomery [28]. The absorbance of the system was measured at 480, 485 and 490 mμ with a Bausch and Lomb Spectronic 20.

As will be discussed later, the yeast cultures studied failed consistently to produce large amounts of the enzyme α-galactosidase when tested with the substrate used as a challenge substrate specific for the presence of the enzyme. The criterion used was 75% transmittance after 15 min. Consequently, soybeans were tested for this enzyme.

Soybean Crude Enzyme Extract

Soybeans (Glycine max, var. Pickett) were allowed to germinate by being placed on wet filter paper. Petri plates were used as the containers and the paper was remoistened 2-3 times daily. Germination was allowed to

continue until root shoots were visible on most of the seeds. The germinated seeds were then weighed en masse and placed in a Waring blendor. Buffer or water was added to fill the blendor completely, allowing no air space. This was done to decrease the amount of foam resulting from the blendor treatment of the soybean seeds. The addition of the suspending menstruum was calculated using the approximate ratio of 10% w/v, that is, for each 10 g of germinated seeds, 100 ml of fluid would ultimately be added. The initial fluid was added to the Waring blendor container for homogenization and the remaining fluid was added later. The system was allowed to homogenize in the blendor for 30 sec at low speed. After treatment, the homogenate was decanted into a large beaker and the remaining fluid was added to satisfy the 10% ratio (as above).

The homogenate was allowed to settle for 30 min, then was strained through cheesecloth to remove the largest particles of the debris. The resulting solution was mixed with enough Dowex 50W-X8 (200-400 mesh) to make the equivalent of a 10% (w/v) suspension. This entire mixture was then filtered through a Whatman No. 1 filter at 4°C. The Dowex apparently acted not only as a purifying agent, but also as a clarifying bed leaving the resultant filtrate acidic and clear. The clarity of the solution was of importance because of spectrophotometric readings.

The clear filtrate is referred to as the crude enzyme extract, and unless otherwise specified, was routinely

stored in dilution bottles at 4°C until used.

Crude Enzyme Extract for pH Studies

When the extract was collected from germinating soybeans for use at varying pH's, it was prepared in a w/v ratio of 10 g/50 ml instead of 10 g/100 ml as previously described (2X stock solution). This 2X extract was combined with a buffer stock which was also at a 2X concentration. The combination of the 2 stock stolutions gave a reciprocal dilution of each to make a series of working solutions of differing pH's. The concentration of material in the resultant series of working solutions is approximately that of a freshly prepared extract of 10% (as above).

All other techniques involved in the preparation of crude enzyme extracts for pH studies are as reported for other crude extracts.

ONPG Stock--α-Galactoside Bonded (α-ONPG)

Two different types of solutions of the substrate ONPG (orthonitrophenyl-1-6-α-D-galactopyranoside, Sigma Chemical Co.) were prepared. The normal working solution of α-ONPG was in a concentration of 2×10^{-3} M in either phosphate buffer at pH 7.0 or in distilled water.

For special situations requiring testing of substrates at different pH's, the α-ONPG was prepared as a 2X stock solution. This special 2X α-ONPG stock solution was then combined with buffer stock of a 2X concentration such that the resultant working solutions of α-ONPG were

reciprocal dilutions of each of the 2 constituent stock solutions.

All stock and normal working solutions were kept at 4°C.

ONPG Stock--β-Galactoside Bonded (β-ONPG)

In some instances it was of interest to parallel the study of α-galactosidase with a study of β-galactosidase. For this reason a working solution of the reagent ortho-nitrophenyl-1-6-β-D-galactopyranoside (β-ONPG, Sigma Chemical Co.) was prepared. Concentrations, preparation and storage were similar to those of the normal working solution of α-ONPG.

Freeze-Thaw Scability

Crude enzyme extracts were placed in Pyrex dilution bottles and frozen at -10°C. The bottles containing these extracts were then thawed at room temperature. A sample from one of the bottles was then assayed for the presence of both α- and β-galactosidase.

This cycle was repeated until the β-galactosidase present in the original preparation showed some degree of inactivation. All remaining extracts were pooled and re-frozen. These samples were then allowed to remain in frozen storage (-10°C) for 8 weeks. After this storage, the extract was again thawed and tested for the presence of both α- and β-galactosidase.

Specific Enzyme Assays

Crude enzyme extract was subjected to an assay for α-galactosidase activity at pH 6.6. α-Galactosidase activity was tested by a modification of the method of Aizawa [4] as reported above under the heading Routine Enzyme Assay. When specific assays were done, spectrophotometric determinations were always conducted as part of the procedure.

The substrate α-ONPG is hydrolyzed by the enzyme α-galactosidase to give galactose and the chromogenic compound o-nitrophenyl, which can be followed spectrophotometrically. One milliliter of working enzyme solution was mixed with 1 ml of 2 X 10^{-3} M ONPG working solution which had been prepared as above from a 2X ONPG stock and a 2X buffer, pH 6.6. A total of 15 such mixtures were incubated at 37°C; sequentially, at 1 min intervals, mixtures were removed and the reaction was stopped by the addition of 2.0 ml of aqueous sodium carbonate. The absorbance of an entire reaction mixture was rea at 400 mμ on a Bausch and Lomb Spectronic 20.

All other specific enzyme assays were conducted in a similar manner. The proportions of crude enzyme extract: ONPG:sodium carbonate was always 1:1:2 with the temperature constant at 37°C. The pH's varied within the range 3.1-8.0. Spectrophotometric readings were always measured at 400 mμ.

Lyophilization

Aliquots of the crude enzyme preparation were placed in each of three pairs of Petri plates. The amounts of solution involved were 10, 15 and 20 ml per plate-pair. These samples were frozen for 24 hrs (-18°C) and then placed in a REPP freeze-dryer (The Vitrus Co., Inc., Gardiner, N.Y.) for 8 hrs. The dried samples were subsequently removed and re-suspended in 5.0 ml of distilled water. Assays were performed before and after treatment for the presence of both α- and β-galactosidase. The procedure was as stated for specific enzyme assays with assay time at 15 min.

Lyophilization studies were also done to determine possible loss of activity due to the freeze-drying technique. The procedure was the same except that the dried material was re-suspended in the same volume of fluid as had been in the original sample. Assays were performed both before and after treatment.

Paper Chromatography

Standard solutions of galactose, glucose, fructose, raffinose, stachyose and sucrose were prepared in concentrations of 1% (w/v). These were used as both challenge and routine solutions for the testing and identification of possible products of enzyme activity. The crude enzyme extract and standard solutions were combined in amounts of 1 ml each and incubated at 37°C. After incubation for 1 hr,

10 λ of each solution was spotted on Whatman No. 1 filter paper for chromatography. The system was subjected to descending chromatography using n-butanol:acetic acid:water (3:3:2) for resolution. This particular solvent system was chosen as it was reported to effect a good separation of raffinose and stachyose [31].

After approximately 18 hrs, the papers were removed and allowed to air dry. The chromatograms were then dipped in the detecting reagent, diphenylamine:aniline:phosphoric acid (5:5:1). The diphenylamine was dissolved in acetone to yield a concentration of 4% (w/v) while the aniline was diluted with acetone to also give a 4% concentration (v/v).

The papers were again allowed to air dry and then heated in a drying oven (about 90°C) for development. Each chromatogram was closely watched and removed when spots began to appear. The time for this phase varied between 5 and 15 min, depending on the temperature of the oven and the number of chromatograms developed at a given time.

Because of the instability of the paper when treated with phosphoric acid and heat, the chromatograms were routinely laminated by commercial process. The laminated chromatograms were then copied for reproduction.

When the crude enzyme extract was challenged with soy milk oligosaccharides, the solutions were spotted on the chromatographic paper at varying times, one series at 5 min intervals (5-30 min total) and a second series at 30 min intervals (30 min-3 hrs total). All other procedures

relating to the treatment of chromatograms were as reported above.

Reproduction of Chromatograms

After lamination, the chromatograms were taken to a commercial photographer. The individual laminated sheets were photographed in order to produce a copy negative. From this copy negative the final photographic prints were made. Although this is an expensive process, no other means provides for excellent reproduction of the original chromatograms with the maximum differentiation of the intensities of the detecting agent. Other means of reproduction can be used when the original chromatogram is not laminated, but when phosphoric acid is included in the detecting agent, lamination is essential for the temporary protection of the chromatogram. Even with lamination, chromatograms so treated will eventually deteriorate.

RESULTS AND DISCUSSION

Preliminary studies were conducted with yeasts as a potential source of the enzyme α-galactosidase to determine if any of the strains tested would be likely sources for large scale production of the enzyme. The results of such studies will be presented here, while those relating to the enzyme as obtained from germinating soybean seeds (Glycine max, var. Pickett) will also be presented.

Yeast Studies

The cultures were maintained on M-12a media as described in Materials and Methods. Table 4 shows the results of the screening test which was used to determine if any of the cultures were acceptable according to the stipulated criteria and thus useful for further study.

As demonstrated, only 2 organisms had the potential for further testing, Candida guilliermondii (strain designation Cand. DGS) and Pichia guilliermondii (strain designation AH 253). The strain used for study was C. guilliermondii, which under test elicited a slightly better production of color after testing with the substrate α-ONPG.

Originally it was anticipated that microbial production of α-galactosidase would necessitate the rapid and inexpensive removal of water from culture supernatants. For

TABLE 4

Strain Designation, Growth and Substrate Response of 20 Yeast Cultures Tested for the Production of α-Galactosidase

Strain	Reactions					
	Growth in Glu-YNB[1]	ONPG	Growth in Glu-YNB	ONPG	Growth in Ra-YNB[2]	ONPG
18	4+	-	4+	-	1+	-
28	3+	-	4+	-	3+	-
29	2+	-	4+	-	3+	-
82	3+	-	4+	-	3+	-
99	2+	-	4+	-	3+	+
140	4+	-	4+	-	1+	-
155	2+	-	4+	-	4+	-
160	1+	-	4+	-	3+	-
173	3+	-	4+	-	3+	-
AH 253	1+	+	4+	-	4+	+
Men 54	1+	-	2+	-	3+	-
Men 95	3+	-	4+	-	3+	-
Men 99	4+	-	4+	-	3+	-
Men 124	2+	-	4+	-	3+	-
M_2 99	4+	-	4+	-	3+	-
NB 2	3+	-	2+	-	1+	-
NS 1067	2+	-	4+	-	1+	-
UM 305	4+	-	4+	-	2+	-
590 Cand.	3+	-	4+	-	3+	-
Cand. DGA	3+	+	4+	-	4+	+

[1]Yeast Nitrogen Base with 0.1% glucose added.

[2]Yeast Nitrogen Base with 1.0% raffinose added.

this reason the culture of C. guilliermondii was grown for comparative studies in several different broths: lactose enriched lactose broth, raffinose enriched lactose broth and whey broth in several different combinations. The latter was chosen because of its plentiful supply, low cost and ease of formulation.

After 2-3 days of growth, the cultures were removed from the shaker, centrifuged to remove the cells and then tested for their α-galactosidase content and subsequent freeze-concentration potential. As a result of freeze-concentration, there was a significant change in the optical density (OD). When lactose enriched lactose broth was used as a culture fluid and tested with α-ONPG the optical density of the solution changed from .0269 (original) to .1308 (freeze-concentration cycle 1) and then to .2676 (freeze-concentration cycle 2).

If the yeast phase of this study had been continued, this concentration by the removal of crystalline water would have been studied further, as it showed some promise as an acceptable method of inexpensive concentration. However, comparisons of α-galactosidase production with residual carbohydrate studies using several different media and several different growth circumstances indicated that these studies failed to provide specific answers to the overall problem of treating the oligosaccharides of soybean milk. In comparison with the results to be reported for germinating soybean seeds, the strains of yeasts tested

appear to be unacceptable as an industrial source of the enzyme α-galactosidase.

As a result of the inability to adequately determine the conditions necessary for the predictable, large-scale production of α-galactosidase from the yeast strains available, it was decided to test germinating legumes, for example Glycine max, for their potential as a commercial source of the enzyme.

Preliminary Screening Tests

Two different legumes were tested, cow peas (Vigna sinensis, var. Purplehull) and soybeans (Glycine max, var. Bragg). Two-day-old germinating seeds were used. The seeds were ground with a mortar and pestle in sufficient phosphate buffer (pH 6.6) to make a liquid. The fluid was decanted and the debris allowed to settle to form a clear supernatant. Samples of 1 ml were removed and tested for the presence of the enzyme α-galactosidase according to the method given as the routine assay procedure.

Additionally, these solutions were filtered through 2 Millipore filters (.45 and .22 μ) to show that the activity was a function of the solution and not any bacterial contaminants of either of the original legume solutions. Table 5 shows the results of these preliminary tests.

As noted, there is little difference between the OD recorded for the original solutions and the same solutions after filtration sufficient to remove any contaminating

TABLE 5

Preliminary Testing of Legumes for Presence of α-Galactosidase

Solution	% Transmittance	OD
Cow pea seeds (*Vigna sinensis* var. Purplehull)		
Extract	5	1.301
Filtrate	4	1.398
Soybean seeds (*Glycine max* var. Bragg)		
Extract	1	2.000
Filtrate	3	1.523

bacteria. It should be noted that the OD in all cases was too intense for definitive testing but was deemed an acceptable recording in screening tests.

The conclusion drawn from these screening tests was that α-galactosidase activity could be recovered from the germinating seeds of these and other legumes as had been reported [1,8,10,14,30,31]. Additionally, it was decided that since the ultimate substrate to be challenged with this α-galactosidase extract was soybean milk, the extract of choice should be generically and specifically similar to the substrate involved. This would obviate any objections to the use of foreign protein, especially protein from microbial sources. This latter, while theoretically and industrially acceptable, might, however, be unacceptable to the maternally protective consumer. For these reasons, soybeans (Glycine max, var. Pickett) were chosen as the source of enzyme for further experimentation.

Extraction Procedures

It was reasoned that the best source of the enzyme α-galactosidase would be those soybean seeds which showed visible germination. The rationale for this assumption is that the substrates of the raffinose family exist in the cotyledons of the seeds as a reserve for ready use during germination. Reports in the literature [1,8,13,31] indicated that such reasoning was acceptable. Consequently, for all further extractions soybeans were allowed to

germinate until shoots were visible on a large majority of the seeds.

The germinating seeds were then processed as reported in Materials and Methods. The ultimate selection of Dowex 50W-X8 (200-400 mesh) was dictated by the needs of the system under study. The enzyme α-galactosidase is active in the acid range. Treatment with the Dowex resin in addition to clarifying the solution effectively acidifies the distilled water extract of the crude enzyme to such a degree that no further acidification is necessary. In addition, although other filter aids, e.g. Celite, were tried, none was as simple to use. Several of those tried not only removed the debris from the extract but also apparently either removed or inactivated the enzyme in the solution.

The proportions of liquid to solid in the extraction scheme were chosen arbitrarily for maximum utilizable yield of crude enzyme extract from any given germination of seeds. The ratio used in this scheme is the one used industrially for the wet grinding of soybean seeds prior to the manufacture of soybean milk [32]. No attempt was made to determine if any other proportions (w/v) were better for functional enzyme yield since experimental results seemed to be acceptable in the proportions used.

Although the original solutions were prepared by the extraction of the enzyme in a buffered suspension, the use of Dowex in the extraction procedure made the use of a

buffer in the acid range superfluous. Moreover, the crude enzyme extracts showed remarkable stability after Dowex acidification since a harvest totaling 350 ml could be stored at 4°C and used for testing over a six-week period with no loss of activity.

Freeze-Thaw Stability

Table 6 shows the results of repeated testing of the crude enzyme extract stability to the stress of freezing and thawing. The extract was challenged with the substrates specific for both α- and β-galactosidase in the manner reported for specific enzyme assays. When the results indicated that the β-galactosidase was considerably inactivated by the repeated freezing and thawing, the extract was left in the frozen state (-10°C) for a protracted length of time. This was done to determine if a long period of frozen storage would have any effect on the solution.

Figure 2 shows a graph of the results listed in Table 6. The readings for α-galactosidase were of more significance since the presence of β-galactosidase was used only as an indicator that freezing and thawing might have an effect on the overall activity of the crude enzyme preparation.

As can be seen from the data, the α-galactosidase remained relatively stable to both the repeated freezing and thawing and to long-term frozen storage. The

TABLE 6

Percent Transmittance and Optical Density after Reaction with α-ONPG and β-ONPG in Studies of Freeze-Thaw Behavior

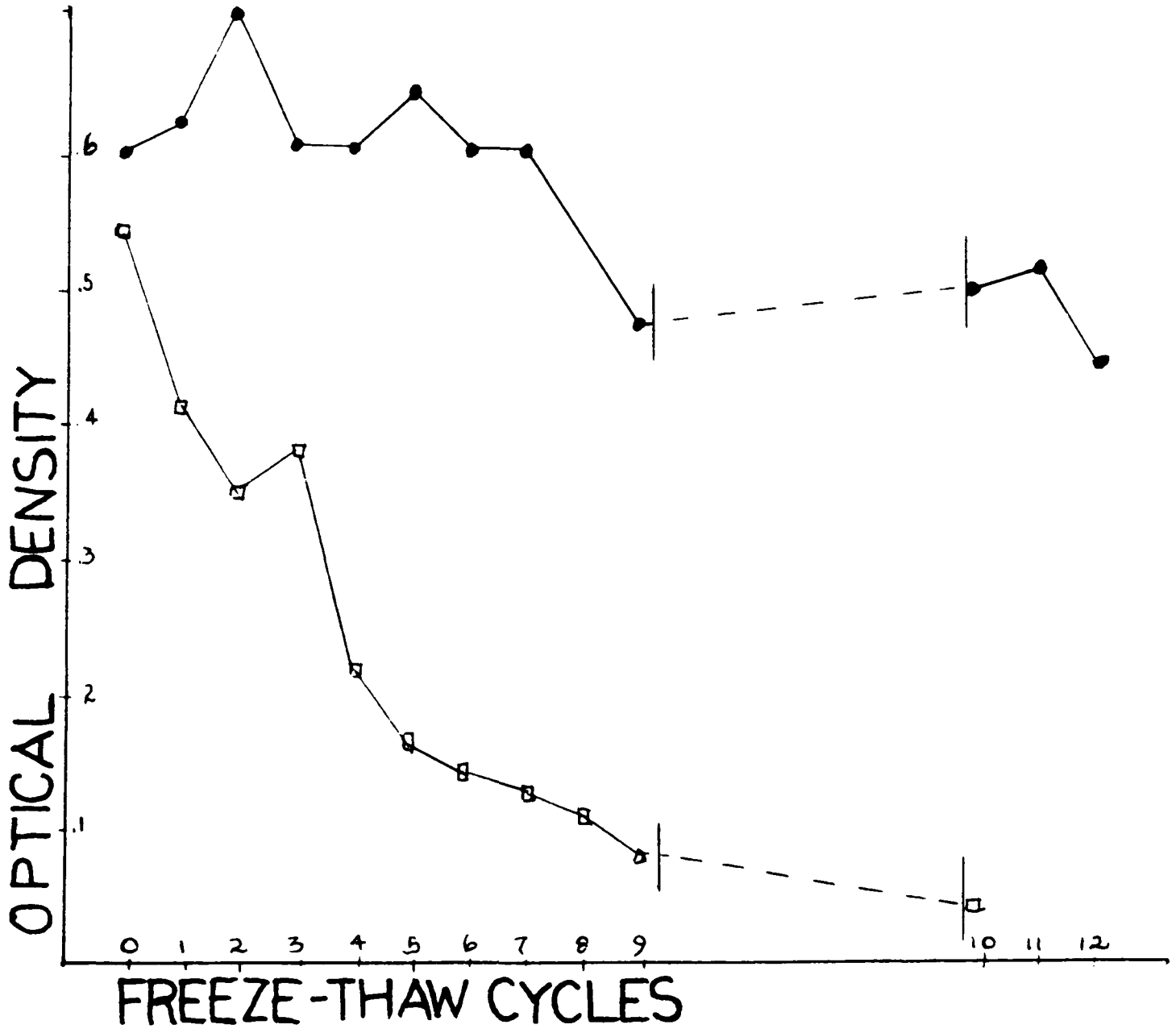
Cycle	α-ONPG		β-ONPG	
	% T	OD	% T	OD
0	25	.602	29	.538
1	24	.620	39	.409
2	20	.699	45	.347
3	25	.602	42	.377
4	25	.602	60	.222
5	23	.638	65	.157
6	25	.602	72	.143
7	25	.602	74	.131
8	30	.523	77	.114
9	34	.469	83	.081
	Six-week frozen storage			
10	32	.495	90[1]	.046
11	31	.509		
12	36	.444		

[1]Assay for β-galactosidase with β-ONPG as substrate terminated at 90% transmittance.

Figure 2. Plot of optical density vs. freeze-thaw cycles for the enzymes α- and β-galactosidase.

●-----● = α-galactosidase

□-----□ = β-galactosidase


OPTICAL DENSITY
.6
.5
.4
.3
.2
.1
0 1 2 3 4 5 6 7 8 9 10 11 12
FREEZE-THAW CYCLES

β-galactosidase, however, showed a consistent downward trend indicative of inactivation. The crude state apparently is not of itself sufficient to protect an enzyme from the repeated effects of freezing and thawing.

The importance of the stability of any commercial preparation to freezing and thawing is obvious. Work hours and schedules must be considered when enzyme preparations are prepared in the industrial laboratory. An enzyme which can be frozen at any stage in the preparation and then thawed subsequently for further processing is preferable to those which must be completely prepared after primary extraction. Complete processing could involve overtime work with the concomitant need for overtime pay. In a highly competitive business, where profit and loss are determined on a few cents per ton, any additional costs must be kept to a minimum. As discussed by Smith and Circle [32] soy products are highly competitive in the market, which precludes any expensive treatments to improve them.

Freeze-Drying (Lyophilization)

Table 7 shows the lyophilization results of the crude enzyme extract aliquots and the re-suspension of these dried preparations. Re-suspension in both the original liquid volume and in a smaller volume were performed to determine if concentration of the enzyme by lyophilization was possible. Because of the freeze-thaw data, inactivation of the α-galactosidase was not expected.

TABLE 7

Percent Transmittance and Optical Density after Reaction with α-ONPG and β-ONPG in Studies of Freeze-Dry Behavior

Vol.	Re-sus.	α-ONPG		α-ONPG (10^{-1})		β-ONPG	
		%T	OD	%T	OD	%T	OD
0	0	3	1.523	63	.201	23	.638
10	10	3	1.523	66	.181	25	.602
15	15	3	1.523	65	.187	23	.638
20	20	2	1.699	64	.194	23	.638
0	0	28	.533				
10	5	3	1.523	67	.174	51	.292
15	5	2	1.699	57	.244	28	.553
20	5	2	1.699	57	.244	39	.409

As the data indicate, the α-galactosidase preparation was stable to the effects of freezing and drying. There was no significant change in the readings for any of the assays of α-galactosidase using α-ONPG as the substrate. It should be noted that the preparation was quite active and the color produced was so intense that a more definitive reading could be obtained when the final reaction mixture was diluted 10^{-1} before spectrophotometric determinations were done.

Analysis of the data in Table 7 shows that the crude enzyme preparation containing β-galactosidase is also stable when challenged with its ONPG substrate after lyophilization and re-suspension. In the case of the β-galactosidase assay it was not necessary to dilute prior to the spectrophotometric determinations as the color produced was not as intense as that produced by the α-galactosidase-substrate reaction. It should be noted that the β-galactosidase assay was included in this series solely for comparison. It was expected that since the enzyme was unstable to freezing and thawing, it would also be unstable to the effects of freezing and drying.

The fact that the enzyme α-galactosidase present in this preparation is stable to freezing and drying allows for further possibilities with regard to its industrial use. If sealed, lyophilized preparations can be stored at room temperature, theh re-hydrated for use at the time required. It is possible to assume that on the industrial scale, the

material could be lyophilized and then later be re-suspended in a small volume of soy milk before being added to the ultimate substrate. Thus large volumes of commercially prepared soy milk could be "pre-digested" with previously frozen or freeze-dried enzyme preparation. This material could then be directly processed or dried into the soy flakes from which commercial soy milks can also be made. If a choice between the 2 possibilities were to be made, then lyophilization might be the method of choice. It, in effect, is both a method of concentration and preservation.

Enzyme Assays

A preliminary assay was run at 1-min intervals for a total of 15 min to determine if the activity of α-galactosidase was rapid enough to permit a short-time interval assay at various pH's. The readings obtained from this assay are presented in Table 8.

The data indicate that there is a rapid change in the optical density of the solution within the 15-min time limit. The rapidity of such a change is indicative of a rapid acting enzyme. According to such data, a 15-min assay could be done routinely to determine the optimum pH for the enzyme system under study. Additionally, this 15-min time frame could of itself be among the criteria of comparison between the α-galactosidase of soybeans and enzymes from other organisms. By inference, any enzyme which

TABLE 8

Preliminary Enzyme Assay

Time[1]	Percent transmittance			OD
	S-1[2]	S-2	Average	
0	100	100	100	.000
1	83	79	81	.092
2	72	75	73	.137
3	66	66	66	.181
4	60	60	60	.222
5	58	54	56	.252
6	47	47	47	.329
7	50	51	50	.301
8	40	43	41	.387
9	38	40	39	.409
10	34	36	35	.456
11	35	37	36	.444
12	31	34	32	.495
13	30	32	31	.509
14	26	29	27	.569
15	22	25	23	.638

[1]Time interval for sampling was 1 min.

[2]S-1 and S-2 refer to Sample 1 and Sample 2.

could not produce such an obvious effect within 15 min would be less desirable as an industrial reagent. It is interesting to note that in only one reported instance of challenge with α-ONPG [24] was any reaction terminated in less than 30 min. The particular enzyme source involved in this report was Diplococcus pneumoniae, a somewhat questionable source for an industrial enzyme for use in the processing of infant hypoallergenic milks.

Based on these preliminary results, a series of assays were then done, using the procedures as outlined to determine the optimum pH and the optimum pH range of activity of α-galactosidase in this crude extract. In order to achieve a range of pH's from 3.2 to 6.7, two different buffer systems were used, a citrate system in the range 3.2 to 5.0 and a phosphate system in the range 4.2 to 6.7.

The data are summarized in Table 9 with graphs of these data in Figures 3, 4 and 5. Figure 3 presents the data for the time interval 0-5 min, while Figures 4 and 5 show the data for the 10 and 15 min time frames, respectively.

In all 3 figures it is evident that the phosphate buffer system at pH 4.6 is the optimum pH for this particular crude enzyme-substrate system. Figures 3 and 4 show a distinct peak at the point on the graph corresponding to the combination of solutions which measures 4.6 on the pH meter. Figure 5 shows a similar peak on the graph but at the 15 min (terminal) time this peak is matched by one

TABLE 9

Results of Enzyme Assay to Determine Optimum Activity as Related to pH and Reported for 5, 10 and 15 Minutes

pH		5 min		10 min		15 min	
		%T	OD	%T	OD	%T	OD
3.2	C[1]	61	.215	39	.409	29	.538
3.5	C	59	.229	40	.398	25	.602
3.7	C	61	.215	39	.409	25	.602
3.8	C	69	.161	45	.347	29	.538
4.0	C	58	.237	42	.377	27	.569
4.1	C	62	.208	46	.337	29	.538
4.2	P[2]	56	.252	30	.523	16	.796
4.4	C	59	.229	38	.420	24	.620
4.6	P	48	.319	24	.620	15	.824
5.0	C	51	.292	29	.538	20	.699
5.1	P	50	.301	31	.509	18	.745
5.5	P	49	.310	32	.495	20	.699
5.8	P	53	.276	38	.420	25	.602
6.0	P	57	.244	39	.409	26	.585
6.2	P	69	.161	55	.260	42	.377
6.5	P	72	.143	62	.208	52	.284
6.7	P	86	.066	75	.125	73	.137

[1]Indicates citrate buffer system.

[2]Indicates phosphate buffer system.

Figure 3. Effect of pH on enzyme activity at 5 minutes

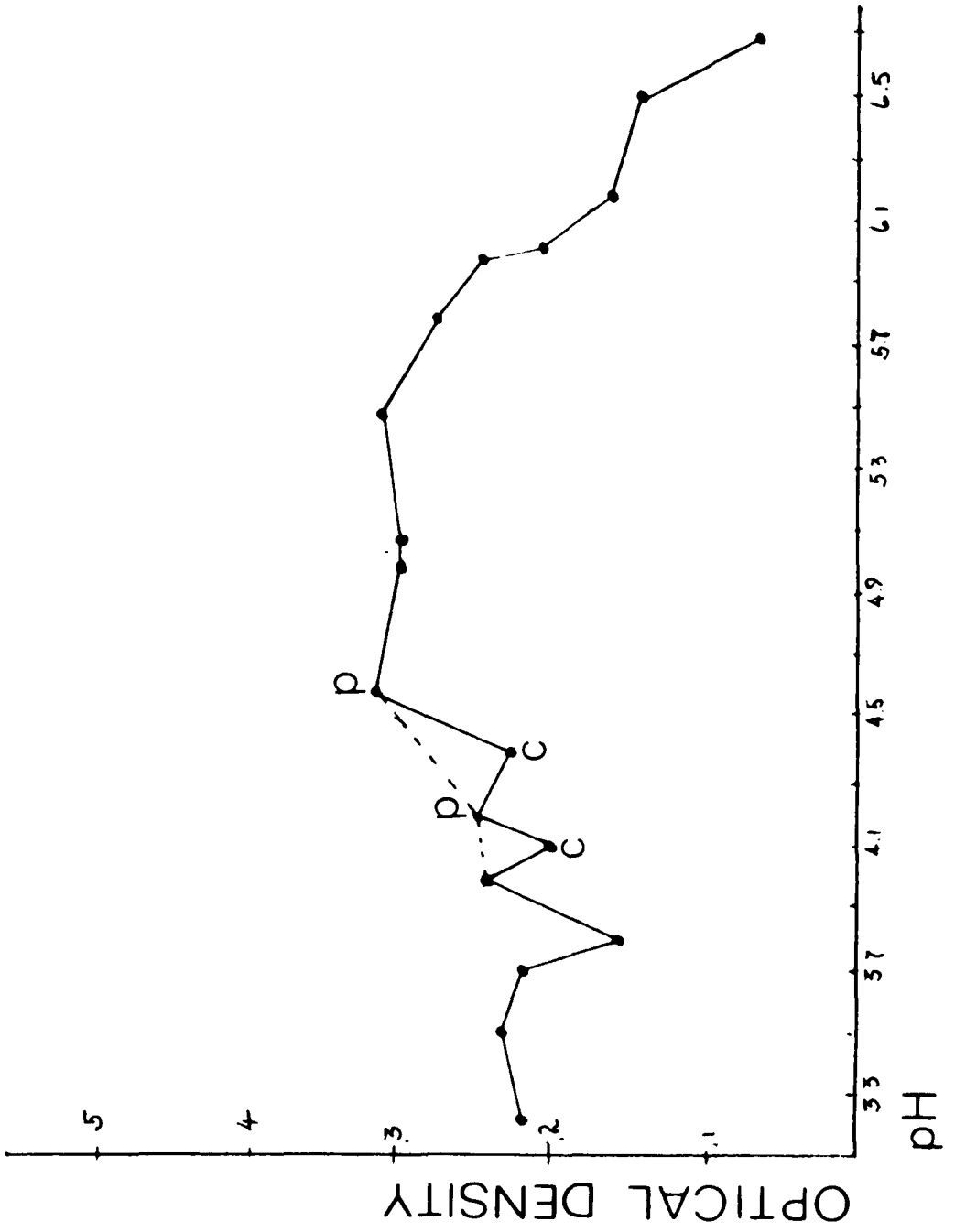
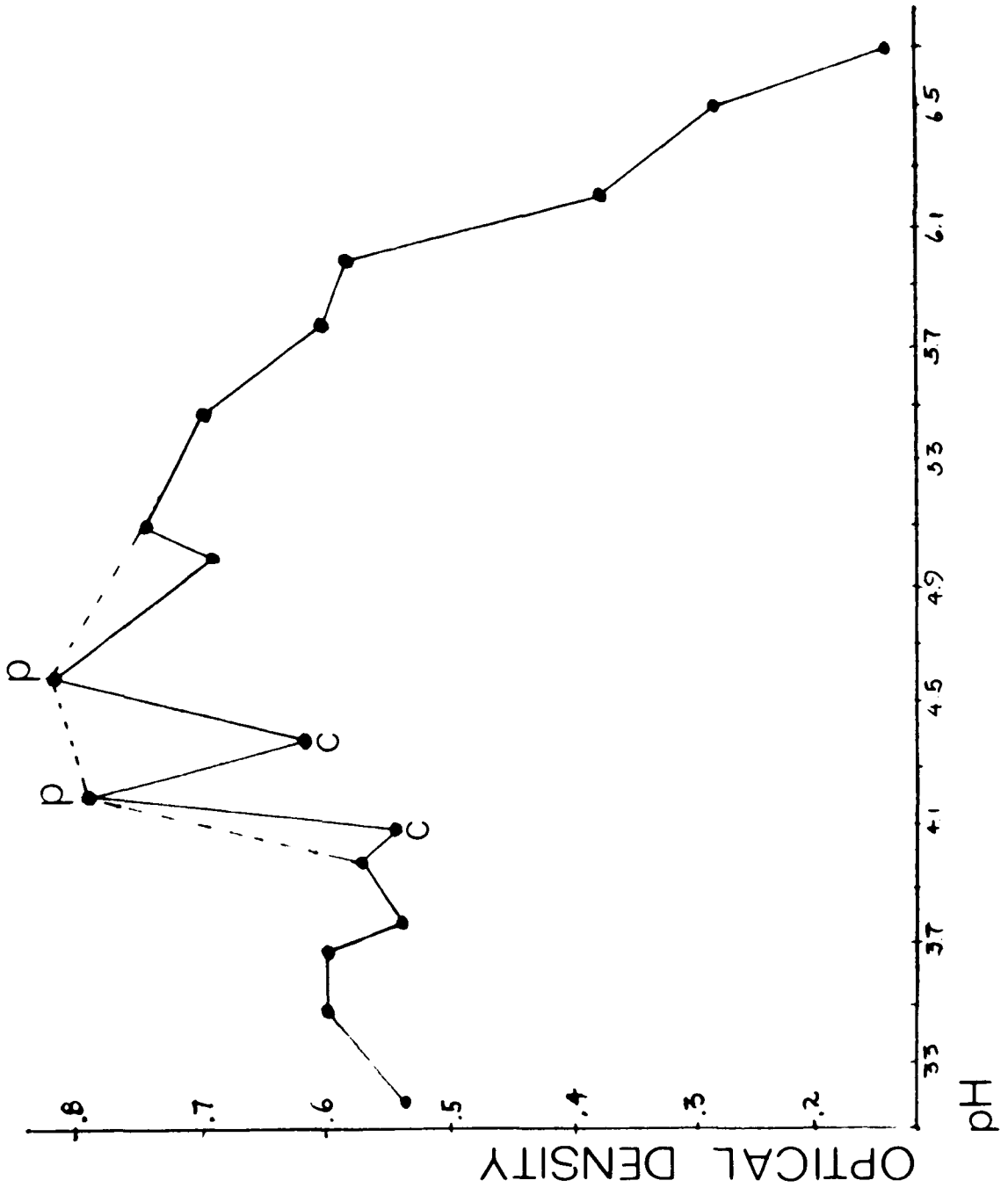

OPTICAL DENSITY
5
4
3
2
1
pH
3.3
3.7
4.1
4.5
4.9
5.3
5.7
6.1
6.5
P
C
P'
C

Figure 4. Effect of pH on enzyme activity at 10 minutes

OPTICAL DENSITY
pH
.1
.2
.3
.4
.5
.6
3.3
3.7
4.1
4.5
4.9
5.3
5.7
6.1
6.5
C
P
C
P

Figure 5. Effect of pH on enzyme activity at 15 minutes


OPTICAL DENSITY
.8
.7
.6
.5
.4
.3
.2
pH
3.3
3.7
4.1
4.5
4.9
5.3
5.7
6.1
6.5
P
C
P
C

similar at the pH 4.2. The conclusion to be drawn is that for routine assays terminating in 15 min, a pH in the range 4.2 to 4.6 would be considered optimum. This same figure also indicates that a pH in the range 4.1 to 5.5 would be acceptable for rapid hydrolysis. The same deductions are acceptable when one studies the graphs of the 5 and 10 min time frames.

It is interesting to note the presence of 3 depressions on these graphs corresponding to 3 points at which the system, although acceptably acid, involved citrate rather than phosphate buffer. These "citrate low-points" tend to indicate that perhaps in the case of this crude enzyme extract optimum pH is a function not only of the actual pH but also of the buffer system involved. The pH 4.4 which is in the optimum range is the best example of a "citrate low-point," a pH in the optimum range which does not behave as expected. This is perhaps an effect of the buffer system rather than the actual pH involved in the enzymatic hydrolysis of α-ONPG. When these observations were first recorded, the assumption was made that the original citrate buffer was incorrectly compounded. After new observations were made, with new buffer, the situation did not change appreciably. Thus the observations were recorded as originally read.

Although several pH's above 6.7 were tested, the results indicated that above this pH the enzyme was unable to function, as activity read spectrophotometrically was

minimal.

Thus readily observable activity occurred in the range 3.2 to 6.7 with the optimum in the range 4.2 to 4.6. This corresponds quite well with the data accumulated on purified and partially purified preparations of α-galactosidase and indicates that the purity of the preparation may have no great effect on the activity of this enzyme as related to pH.

Chromatography Studies

The test of the acceptability of any system is based on whether or not the system will accomplish what it is reputed to do. In the case of α-galactosidase and the system proposed for its use, chromatographic studies were done to determine if commercial substrates, challenged with the enzyme preparation, would respond in the assumed manner.

Figures 6, 7, 8, 9, 10 and 11 are photographs of duplicate chromatograms done as reported. Figures 6 and 7 are pictures of chromatograms spotted to determine the relative mobilities of known and suspected substrates and products. Each spot (2 through 8) on the chromatograms shown as Figures 8, 9, 10 and 11 were done at different time intervals in an attempt to develop a picture of the carbohydrates extant in the enzyme-substrate system at these different times.

Figure 6. Photograph of a chromatogram developed for 18 hours showing the mobility of the standard solutions. Abbreviations are as follows: ST = stachyose, RA = raffinose, SU = sucrose, GL = glucose, GA = galactose, FR = fructose.


GA
GL
RA
SJ
SU

Figure 7. Photograph of a chromatogram developed for 18 hours showing the mobility of the standard solutions. In addition to the abbreviations previously mentioned, Fb = the sugar referred to as fructose (b).

Figure 8. Photograph of a chromatogram developed for 18 hours showing the mobility of the sugars extant in the test solutions. Sample 1 indicates the carbohydrate input of the system, sample 2 is the substrate solution and samples 3 through 8 were spotted at 5 min intervals. The test was terminated at 30 min. All abbreviations are as previously listed.

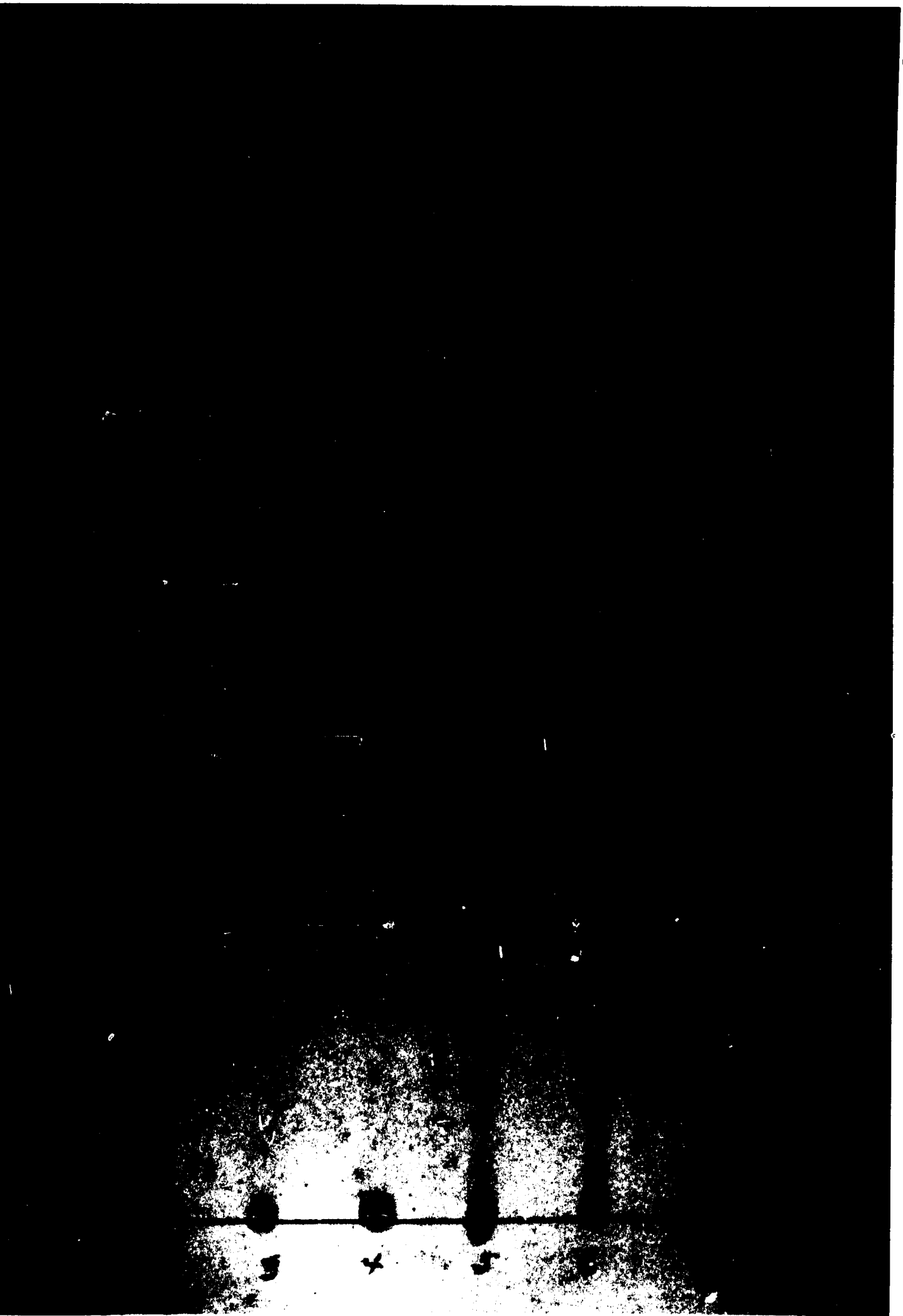


Figure 9. Enzyme-substrate challenge terminated at 30 min, with test solutions sampled at 5 min intervals. All conditions are as cited for Figure 8.

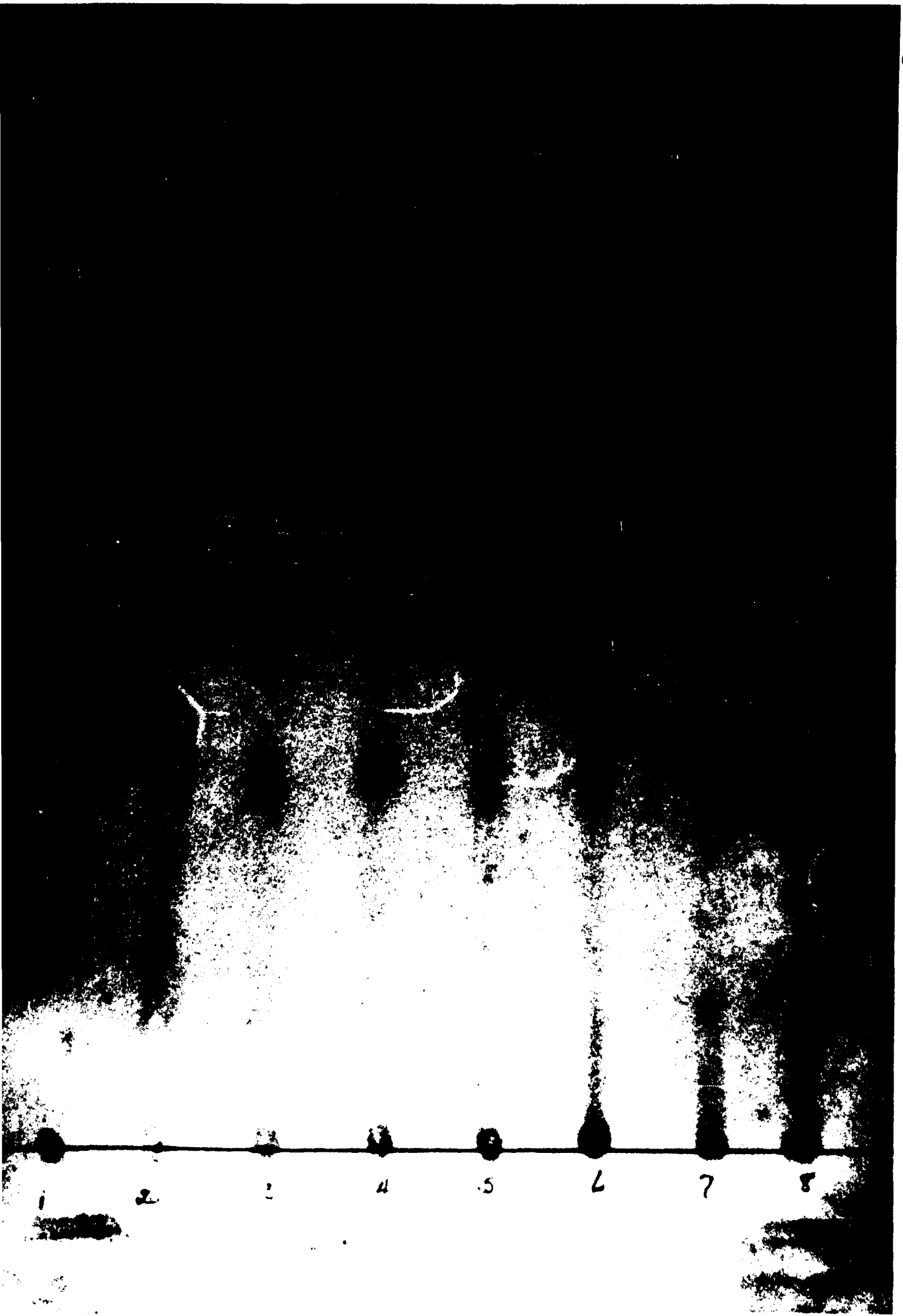
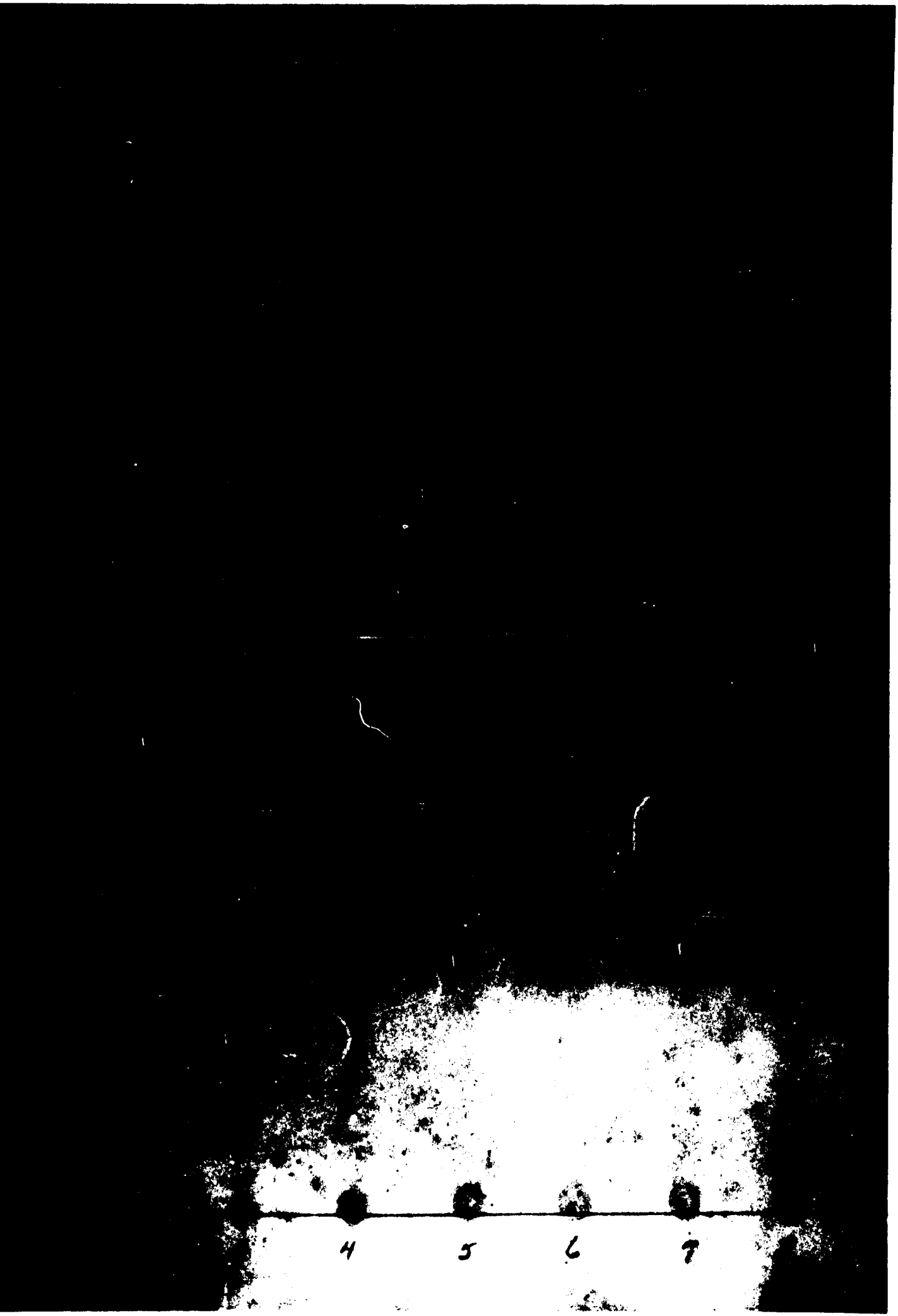

1
2
3
4
5
6
7
8

Figure 10. Photograph of a chromatogram developed for 18 hours showing the mobility of the sugars extant in the test solutions. Sample 1 indicates the carbohydrate input of the enzyme system, sample 2 is the substrate solution and samples 3 through 8 were spotted at 30 min intervals. The test was terminated at 3 hrs. All abbreviations are as previously noted.

Figure 11. Enzyme-substrate challenge with test solutions sample at 30 min. Test terminated at 3 hrs. All notations the same as for Figure 10.


4
5
6
7

Consistent on all photographs are grey-white streaks resembling veins of marble which appear sporadically on the face of these photographs. These are artifacts due to the lamination process which was used to preserve the chromatograms for photography.

Figures 6 and 7 are pictures of the relative positions of the probable digestion products of any treatment of an extract of soy milk oligosaccharides (substrate) by a mixture of the enzymes α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase. The galactosidases mentioned were previously demonstrated as present in the preparation. The glucosidases were hypothesized as probably present in this crude enzyme extract. The hypothesis is regarded as reasonable in the light of reports from the literature [1,10] for somewhat analogous enzyme extracts.

The chromatograms as pictured in Figures 6 and 7 show the following carbohydrates as spotted in the following positions: (1) fructose, (2) galactose, (3) glucose, (4) raffinose, (5) stachyose, (6) sucrose. These mono-, di- and oligosaccharides are the major substrates and products of the scheme in which a mixture of enzymes would act.

Spots (7) and (8) on the chromatograms pictured in Figures 6 and 7 are mixtures of these standard solutions. Position (7) was spotted with a sample of a mixture containing fructose, glucose and sucrose. The spot located at position (8) was a sample from a mixture containing

galactose, raffinose and stachyose. As can be noted from the photographs, the spots in the mixtures are lighter in intensity and smaller in dimension than those of the original standard solutions. This reflects the fact that the mixtures were dilutions of the original standard solutions. The fact that this difference in intensity can be detected in both the original chromatograms and the photographs of these chromatograms is of some importance. It would not be in order to draw inferential conclusions from differences in intensities unless it could be demonstrated that these differences in intensities do exist as detectable to the eye.

Of considerable interest in Figure 8 is the presence of 2 spots resulting from the sample spotted at position (1). The more migratory of the 2 spots corresponds to the behavior of fructose as seen by comparison with spot (1) on Figure 7. The other spot, less migratory in nature, does not appear on Figure 7 in a corresponding location. A careful study of the 2 chromatogram pictures results in the observation that the intensity of the fructose spot in Figure 7 is not equaled by either of the spots at position (1) in Figure 8. The sample in Figure 8 has, in effect, been diluted. For purposes of discussion, this second spot will be referred to as fructose (b).

The spot known as fructose (b) (Figure 8) would be of little importance except for the fact that consistently, in an intensity (concentration) which increases with time,

this same spot appears on the other chromatogram pictures done after challenging the substrate with enzyme extracts. Consequently the identity of this unknown is of some importance.

The evidence as to the identity of fructose (b) is that (1) it appears in what is a standard solution of a reagent grade of fructose; (2) it migrates more rapidly than raffinose, a trisaccharide, or stachyose, a tetrasaccharide; (3) it migrates more slowly than does galactose, glucose or fructose, three different monosaccharides; and (4) if lines are drawn parallel to the movement of the solvent front, mono-, di- and trisaccharide "belts" can be formed. The fructose (b) lies in the same "belt" as sucrose, a disaccharide. This same spot appears as a result of enzymatic reaction where there is known to be at least 2 and probably more enzymes present. For these reasons the spot could be analyzed as either melibiose or a disaccharide composed of fructose. Such analysis is consistent with both known facts and current hypotheses.

One of the difficulties with the identification of the unknown spot on the chromatograms is that the literature cited does not contain a published chromatogram using the butanol:acetic acid:H_2O (3:3:2) solvent system after the treatment of legume oligosaccharides with α-galactosidase. Those chromatograms which have been published use a different solvent system. However, they do show a spot, consistently, which was determined to be melibiose. In

these published chromatograms, the melibiose spot is shown as migrating between sucrose and raffinose. This is characteristic of the unknown reported here. However, when melibiose was tested at 18 hrs, it failed to migrate to the location occupied by the unknown. Therefore, in discussing this spot on other chromatograms it will be referred to as fructose (b) with the reservation that it could be a disaccharide composed of fructose.

Figures 8 and 9 are pictures of chromatograms in which spots (3) through (8) are samples from a reaction system involving soy milk substrates and enzyme extract. The spots were placed on the paper at 5 min intervals giving rise to a developed picture of the first half-hour of the enzyme-substrate reaction. Each row of developed sugars has been labeled with the following abbreviations: (RA) for raffinose, (ST) for stachyose, (Fb) for the sugar referred to as fructose (b), and (GGS) for the very dark spot which represents the confluence of galactose, glucose and sucrose.

A comparison of the substrate spot (2) with patterns (3) through (8) shows the accumulation of the products raffinose, fructose (b) and galactose, glucose and sucrose (in the confluent complex) at the expense of the oligosaccharide complex in the substrate represented by the heavier streak at locus 2. This "oligosaccharide streak" is less apparent in the spots developed after the time interval reactions, as can be noted by a gradual clearing of this

area. Both pictures show the same pattern although certain features are more visible on one or the other photograph.

The spot shown as (1) has been included on all chromatograms as the pictorial representation of the carbohydrates contained in the crude enzyme extract. It should be noted that there is a small amount of stachyose involved as input into the system. This small amount of oligosaccharide involved as input, however, is outweighed by the amount which stains more intensely in location (2) as being present in the substrate.

The accumulation of stachyose and raffinose within the 30 min interval as represented by Figures 8 and 9 is evidently the result of the clearing of the "oligosaccharide streak" by the hydrolysis of terminal galactosides first on the larger oligosaccharides to yield stachyose and galactose and then on the stachyose to yield raffinose and galactose.

The increasing intensity and size of the spots referred to as fructose (b) and GGS are also consistent with the above analysis. Thus Figures 8 and 9 indicate that as early as 5 min into the time frame, hydrolysis of the 1-6-α-D-galactoside terminals of soy milk oligosaccharides has occurred with the concomitant result that smaller and presumably more digestible residues are produced.

Figures 10 and 11 represent similar studies, but with a different time frame and different time intervals. Spots (3) through (8) on these chromatograms were done at

30 min intervals starting at 30 min and terminating at 3 hr. Again spot (2) is the substrate under test and spot (1) represents the carbohydrate input of the enzyme preparation. The extremely dark edges which occur on the right side of Figure 10 and the left side of Figure 11 are not loci of carbohydrates but rather artifacts due to the development of the chromatograms in an open heat system. Since this problem had been noted in the development of early chromatograms, it was decided that all chromatogram-pairs would be positioned alternately for heat development. This alternative positioning had the effect of preventing the same edge of each chromatogram-pair from being darkened beyond acceptability.

In studying the photographs labeled as Figures 10 and 11 the same trend as discussed previously is again apparent. The "oligosaccharide streak" is even more obvious than in Figures 8 and 9. The pattern of the clearing of this streak is evident after 1/2 hr (spot 3) and continues through 2-1/2 hrs (spot 7). Only at 3 hr (spot 8) is a reversal of this trend indicated. For the first time there appears to be some evidence for the reversibility of the reaction with the slight darkening of a spot corresponding to one of the substrate oligosaccharides.

The substrates stachyose and raffinose (ST and RA) lose intensity, indicative of a degradative attack and there is an increase of the intensity of the GGS complex and the spot known as fructose (b) with time.

milks, the addition of enzyme after the wet grinding process but before canning and retorting would be both simple and effective.

When soy milks are made from full-fat soy flour the process is a little more complex. The flours must first be re-hydrated from the dried soy flour or flakes and then mixed thoroughly for maximum dispersibility of the re-hydrated materials. The proposed enzyme system could still be easily added after re-hydration.

In either case, pH would not be a significant problem since enzyme could be added to the system, the pH lowered for maximum activity and then raised prior to the processing of the milk.

Recent research into the development of off-flavors during the grinding of raw legume seeds [20] has indicated that the lipooxidase system is incriminated in the development of off-flavors. This system can be effectively controlled by the wet grinding of legume seeds at a low pH with the addition of an antioxidant such as α-tocopherol or citric acid. Coincidentally, at this lower pH maximum protein extraction was obtained. One proposal, then, for maximum digestibility of a high protein product would be to wet grind germinating soybean seeds at a low pH in the presence of an acceptable antioxidant and then raise the pH to that level needed for the action of added crude enzyme extract. Maximum conversion of oligosaccharides would be favored by such a scheme. After a calculated time

The structure of the GGS complex is of interest on these photographs, for the leading edge of this confluence is indicative of glucose, while the trailing portion is composed of sucrose. Galactose is in the center of the complex. Thus, the location of the area of greatest intensity is of importance in the determination of which products have accumualted with time. In the ultimate, regardless of the exact identity of the smaller sugars, there is evidence that cleavage has occurred with the dual result of decreasing the amounts of oligosaccharides present and increasing the amounts of mono- and disaccharides present.

After 2-1/2 hrs of treatment the intensity of the stachyose spot begins to change, indicating the resynthesis of oligosaccharides. The nature of this reaction was not investigated as it was not considered germane to the problem under study. Its relation to the degradation of the soy milk oligosaccharides is that it gives some indication as to the need for the timely addition of more oligosaccharide substrate to maintain the K_{eq} in the forward direction favoring hydrolysis.

The development of any method for the pre-digestion of the undesirable oligosaccharides in soy milk should take into account current and currently proposed methods for soy milk production. According to Smith and Circle [32], soy milk can be processed from whole soybeans or full-fat soy flour. If whole soybeans are used in the production of soy

milks, the addition of enzyme after the wet grinding process but before canning and retorting would be both simple and effective.

When soy milks are made from full-fat soy flour the process is a little more complex. The flours must first be re-hydrated from the dried soy flour or flakes and then mixed thoroughly for maximum dispersibility of the re-hydrated materials. The proposed enzyme system could still be easily added after re-hydration.

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interval, the pH could be raised still further, to that of the ordinary product.

Such a scheme would involve a minimum outlay of capital investments, improve the quality of the product with simple, easy, repeatable methods and not add significantly to the consumer cost. The cost of the hypoallergenic soybean milk to the American consumer is not of major significance since the product is only used when medically necessary. Cost is relevant on the world-wide scale, particularly to the financially and nutritionally emerging nations. A more desirable world-wide product could result in fewer nutritional problems and protein deficiencies. Anything which could reduce the specter of infant kwashiorkor is of a value which would outweigh a slight additional cost.

SUMMARY

Intestinal flatulence is reputed to be a usual sequel to the ingestion of legumes of the bean family. The treatment of soy milk oligosaccharides by purified preparations of the enzyme α-galactosidase from microbial or fungal sources is an experimentally sophisticated but relatively expensive means of "pre-digesting" the oligosaccharides incriminated in this flatulence.

Evidence presented in this paper indicates that the removal of these α-bonded residues from soy milk oligosaccharides can be accomplished by the use of a homogeneric extract of enzymes obtained from the germinating seeds of the soybean, Glycine max, var. Pickett.

The advantages of such a scheme are many. The ease of extraction is an important factor in the industrial use of an enzyme. This particular enzyme extract is easily prepared with simple means. The most expensive item in the extraction scheme is the Waring blendor.

Microbial and fungal α-galactosidases are "pre-digesting enzymes" involved in the splitting of oligosaccharides prior to their entrance into the cell. Thus, they are exoenzymes, produced in the cell for use outside the cell. If the enzyme α-galactosidase were found in intimate

association with the cell, the problems involved in harvesting would be more easily solved. However, when microbial and fungal organisms are cultured, the enzyme α-galactosidase is found in the medium and must be greatly concentrated prior to use.

The same enzyme, when collected from germinating seeds, is found in the seed cotyledon. Thus, its location is already marked and concentration, if required, is much easier.

Industrial work schedules require that the materials involved must be prepared for possible storage and later use. Any enzyme system which must be handled with extreme skill and care is not an acceptable possibility for the industry to consider useful. It lacks the requirement of flexibility which would indicate utility in the industrial arena.

The enzyme preparation containing α-galactosidase from the germinating seeds of the soybean possesses the ability to be stored in the frozen state and thawed prior to use with no loss of activity. This material can be refrozen and rethawed. This permits some degree of employee flexibility with no loss of material, both factors resulting in less monetary output.

The optimum conditions for the proposed scheme remain to be determined. These conditions will vary from plant to palnt, depending on the means of soy milk production. The fact remains that this scheme has a greater

potential for the improvement of the commercial soy milks than do other schemes proposed in the past few years. It is quicker, cheaper, and simpler to prepare and probably as effective to use. Its sophistication lies in the fact that the preparation is homogeneric.

The homogeneous preparation is the preparation of choice for studies of molecular weight, amino acid profile, and the important properties of enzymes related to their comparative kinetics. Although these properties are interesting to the food technologist, they are not critical to the important technological test, industrial functionality.

The homogeneric enzyme preparation discussed in this paper is a functional preparation which has the added advantage of being a rapid and inexpensive means to a desirable end. It has the added enhancement of not adding any foreign proteinaceous substances to a product designed primarily for an infant already suffering from an allergenic response.

Whether this scheme will be adopted by industry remains to be seen. It offers an alternative to the currently proposed methods of solving the problem of flatulence in soybean preparations. With a world-wide protein crisis, which promises to become greater, there is a strong possibility that soybeans will not be the only legume used for the production of protein.

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VITA

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Miss Crocco has taught on the secondary and collegiate levels in Maryland, the District of Columbia and Louisiana. She has additionally worked with the post-secondary education of the vocationally-oriented under cooperative programs established by the State of Maryland and the United States Department of Health, Education, and Welfare.

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EXAMINATION AND THESIS REPORT

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Title of Thesis: Treatment Of Soy Milk Oligosaccharides By A Homogeneric Enzyme Extract Containing α-Galactosidase

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